ANTIBODIES SPECIFIC FOR INTRACELLULAR DOMAIN OF PROTEIN TYROSINE PHOSPHATASE

FIELD OF THE INVENTION

The present invention relates to antibodies that specifically bind to intracellular domains of two or more kinds of protein tyrosine phosphatases (hereinafter referred to as PTPs) and to methods for generating the same. More particularly, the present invention relates to antibodies having specificity to intracellular domains of PTPs (e.g., LAR (leukocyte antigen related molecule) and CD45), which are useful for analysis and quantitative determination of PTPs, for identification, detection, and isolation and purification of novel PTPs, and for development of medical drugs applicable to treatments such as therapy, prevention and alleviation as well as diagnosis of the disease states associated with insulin resistance.

BACK GROUND OF THE INVENTION

Mechanisms involving in the onset of arteriosclerosis have been gradually elucidated in these days, and risk factors thereof have been identified. Especially, hypercholesterolemia, hypertension, diabetes, and smoking are recognized to be manifest four risk factors, thus the therapeutic treatments have been extensively carried out. Clinically common pathology of these disease states is insulin resistance. The meaning of insulin resistance is nearly equivalent to the reduction of sensitivity to insulin in cells, and may be that the actions of insulin upon the uptake of sugar into the cells are deteriorated. The insulin resistance may be caused due to the abnormalities in secretion of insulin itself, abnormalities of insulin receptors on target cells, abnormalities of an intracellular signaling system, and reduced supply of sugar to the tissue based on peripheral circulatory disorder that is caused hemodynamically. Reaven, in 1988, reported that many pathological states

are developed due to the insulin resistance, and designates a pathological state as "syndrome X" that may concurrently represent insulin resistance, glucose tolerance abnormalities, hyperinsulinemia, hypertriglyceridemia, hypo-HDL cholesterolemia and hypertension, and further suggests that the pathological state syndrome X closely participates in the onset of arteriosclerosis (Reaven, G. M. *et al.*, *Diabetes*, **37**, 1595-1607, 1988).

In addition, sugar supply to the cells is known to be generally decreased through insulin resistance, accompanied by enhancement of insulin secretion from pancreas, thus leading to hyperinsulinemia. Therefore, several problems in connection with insulin resistance have been raised in clinical fields. For example, insulin resistance and hyperinsulinemia are reported to promote diabetic nephritis (Niskanen, L. *et al.*, *Diabetes*, **41**, 736-741, 1993), and to elevate frequency of diabetic retinopathy (Yip, J. *et al.*, *Lancet*, **341**, 369-370, 1993). Moreover, insulin resistance has been reported to enhance plasminogen activator inhibitor 1 (PAI-1), to deteriorate the functions of a blood fibrinolytic system (Potter van Loon BJ *et al.*, *Metab. Clin. Exp.*, **42**, 945-954, 1993), and to trigger arterial atherosclerosis (Sato, Y. *et al.*, *Diabetes*, **38**, 91-96, 1989).

Prevalence rate of diabetes accounts for 5% of the total population, and approximately six millions of Japanese citizens are suffering from diabetes. Diabetes comprises insulin dependent diabetic mellitus (IDDM) and non-insulin dependent diabetic mellitus (NIDDM). Reportedly, IDDM accounts for about 7% of the total diabetes cases, whilst NIDDM does about 90%. In particular, a significant causative factor of the onset of NIDDM that corresponds to a majority of diabetes has been conceived as the insulin resistance.

To date, tyrosine phosphorylation of intracellular proteins has been elucidated to play important roles in signal transduction of insulin. Insulin receptor is a hetero-tetramer of two glycoprotein subunits, namely an α-subunit having a molecular

weight of 135 kDa and a β-subunit having a molecular weight of 95 kDa, which are bound through disulfide bonds resulting in a hetero tetramer having an α2β2 structure. The α-subunit has an insulin binding activity, while the β-subunit has a protein tyrosine kinase (PTK) domain that is activated by autophosphorylation. Accordingly, when insulin is bound to the α-chain of an insulin receptor, certain tyrosine residues existing in the β-chain of the insulin receptor are autophosphorylated by tyrosine kinase activity of the receptor. The activity of insulin receptor tyrosine kinase is further promoted through the tyrosine autophosphorylation. It is reported that thus activated insulin receptor tyrosine kinase phosphorylates tyrosine residues of IRS (insulin receptor substrate), the intracellular substrates thereof, and signal transduction is proceeded through recognition and binding of the tyrosine-phosphorylated insulin receptors by Ash/Grb2 or PI-3 kinase, finally resulting in manifestation of biological activities of insulin, such as glucose uptake, sugar metabolism and cell proliferation (see, Fig. 9, Goldstein, B.J. *et al.*, *Receptor*, **3**, 1-15, 1993; and Kanai, F. *et al.*, *Biochemical and Biophysical Research Communications*, **195** (2), 762-768, 1993). In this signal transduction pathway, however, an enzyme that inactivates the activated insulin receptors, i.e., PTP, which is a protein tyrosine phosphatase, has not been progressively studied.

Moreover, fundamental mechanisms for lymphocyte activation, proliferation, differentiation, cell death and the like, which are also ingeniously controlled by tyrosine phosphorylation have not been likewise elucidated. Studies on signal transduction of lymphocyte in light of PTK have been mainstream of the studies so far, however, analyses from PTPs have also been performed, and importance of studies from both aspects have been manifested.

The serious studies of PTPs were initiated after completion of cloning of PTP1B gene and elucidation of the nucleotide sequence thereof by Fischer *et al.* in 1988, which is cytoplasm type PTP derived from human placenta (Tonks, N. K. *et al.*, *J. Biol. Chem.*, **263**,

6722-6730, 1988; Charbonneau, H. *et al.*, *Proc. Natl. Acad. Sci. USA*, **85**, 7182-7186, 1988). Consequently, homology to PTP1B could be observed not with the known serine/threonine phosphatases but with two cytoplasmic regions of CD45, a transmembranous molecule involved in a hemopoietic system. Moreover, CD45 was also revealed to have PTP activities (Tonks, N. K. *et al.*, *Biochemistry*, **27**, 8695-8701, 1988; and Charbonneau, H. *et al.*, *Proc. Natl. Acad. Sci. USA*, **85**, 7182-7186, 1988).

It is presumed that there are as many as 500 PTP genes in human, and to date, not less than 80 kinds of PTPs were cloned based on their homologies of cDNA sequences, while new PTPs have been still reported subsequently (Streuli, M. *et al.*, *J. Exp. Med.*, **168**, 1523-1530, 1988; Krueger, N. X. *et al.*, *EMBO J.*, **9**, 3241-3252, 1990; and Trowbridge, I. S. *et al.*, *Biochim. Biophys. Acta*, **1095**, 46-56, 1991). The PTPs that form a superfamily are broadly classified to three families. Namely, there are three groups, PTP, DS-PTP (dual-specificity-PTP) and LMW-PTP (low molecular weight-PTP). Homologies between primary structure of each of the family members are not that high, and in particular, no homology was suggested between PTP and LMW-PTP except for the homology of both enzymatic active centers, however, based on the studies on crystallography, it was illustrated that the molecules belonging to these families exhibit surprisingly common characters in their tertiary structures (Fauman, E. B. *et al.*, *Trends Biochem. Sci.*, **21**, 413-417, 1996). Furthermore, PTPs can be classified generally to: (1) receptor type (or, membrane type) PTPs having transmembrane region (LCA, leukocyte common antigen, namely CD45, LAR and PTPs α, β, γ, δ, ε and ζ); and (2) cytoplasm type PTPs without transmembrane region (PTP1B, TC-PTP, PTP-MEG, PTPH1, STEP, PTP1C, FAP1, SHP1, SHP2, PEP, PTP-PEST and the like).

Many of receptor type PTPs have two PTP homologous domains inside the cell (domain 1 and domain 2, see, Fig. 1(a) and (b)). A sequence comprising cysteine (signature motif), Ile/Val-His-Cys-Xaa-Ala-Gly-Xaa-Xaa-Arg-Ser/Thr-Gly (SEQ ID NO:

2), has been conserved in the phosphatase domains between the PTPs reported heretofore. The researches on crystallography of PTP1B revealed that the region forms small pockets on the surface of a PTP molecule, and that the cysteine residue is located to the bottom of the pocket, participating directly in binding of the molecule to phosphate (Barford, D. *et al.*, *Science*, **263**, 1397-1404, 1994). In addition, it was also revealed that the depth of the pocket of PTP may determine the specificity of serine/threonine phosphatase because phosphate that is binding to serine or threonine cannot reach to the pocket of the enzymatic active center of PTP1B. Moreover, the importance of the above-mentioned signature motif in exhibiting the enzymatic activity has been elucidated from experiments of the mutants (Streuli, M. *et al.*, *EMBO J.*, **9**, 2399-2407, 1990). Taking into account of these observations, it has been conceived that the conserved cysteine in domain 1 may play an important role in exhibiting the enzymatic activity, and domain 2 may determine the substrate specificity of the enzyme.

Receptor type PTPs have two or one intracellular enzymatic region(s), and they can be classified to several groups in accordance with their properties of extracellular domains. There are: CD45 having one extracellular fibronectin type III domain and being highly modified with sugar chains; LAR, PTPδ and PTPσ having Ig-like domains and fibronectin type III domains; PTPμ and PTPκ having MAM (meprin, A5 antigen, PTPμ) at N-terminal ends; PTPγ and PTPζ having a carbonate dehydratase domain at N-terminal ends; and PTPα and PTPε having short extracellular domains, and any of these PTPs has two enzymatic regions. Meanwhile, any of PTPs having single enzymatic region for example, PTPβ, and CD148 (PTPη, DEP·1 and the like), is constituted from only fibronectin type III domains in its extracellular domain.

Cytoplasm type PTPs have one enzymatic region in principle, and have been classified to several groups based on their properties of non-enzymatic regions. Cytoplasm type PTPs may have SH2 region, PEST region, and band 4.1 region. DS-PTPs

are enzymes that dephosphorylate serine or threonine as well as tyrosine residue, which may include Cdc25, MAP kinase phosphatase, VH-1 and the like. LMW-PTP is constituted from an enzymatic region alone, and its molecular weight is reported to be about 18 kDa.

Among the groups of PTPs, LAR derived from human is a prototype of receptor type protein tyrosine phosphatases, which was cloned from human placental genome library using a phosphatase domain of CD45, a receptor type protein tyrosine phosphatase, as a probe (Streuli M. *et al.*, *J. Exp. Med.*, **168**, 1553-1562, 1988). CD45 is specifically expressed on hemocytic cells, whilst LAR is expressed on the cells other than hemocytic cells, particularly in insulin sensitive organs such as liver and skeletal muscle (Goldstein B. J., *Receptor*, **3**, 1-15, 1993). LAR is especially interesting among many types of receptor type PTPs due to its similarity of the extracellular domain to cell adhesion molecules. The entire structure of LAR is elucidated as having 150 kDa of extracellular E-subunit that consists of Ig-like domains and fibronectin type III domains, and 85 kDa of P-subunit (phosphatase subunit, set out in SEQ ID NO:1) comprising a transmembrane region and an intracellular domain having two phosphatase domains, which are covalently bound immediately outside of the cell membrane (see, Fig. 1, Streuli M. *et al.*, *EMBO J.*, **11**, 897-907, 1992). Additionally, LAR constitutes a subfamily together with PTPδ and PTPσ, and exists in peripheral portions of desmosomes (junctions with extracellular matrix through integrin) and in adherens junctions (intercellular adherent portions through cadherin), (Serra-Pages, C. *et al.*, *EMBO J.*, **14**, 2827-2838, 1995; Pulido, R. *et al.*, *Proc. Natl. Acad. Sci. USA*, **92**, 11686-11690, 1995; Kypta, R. M. *et al.*, *J. Cell Biol.*, **134**, 1519-1530, 1996; and Aicher, B. *et al.*, *J. Cell Biol.*, **138**, 681-696, 1997).

A large number of functional roles of LAR have been reported to date. For example, it was reported that: responses to neurotrophin are decreased in LAR deficient nerves (Yang, T. *et al.*, 27th Annual Meeting of the Society for Neuroscience, New

Orleans, Louisiana, USA, October 25-30, 1997, *Society for Neuroscience Abstracts*, **23**, 1-2, 1997); LAR homologues of Drosophila are predominantly expressed in nervous system, and the deficiency thereof results in delay of timely segregation of motor axon from nerve fascicle (Krueger, N. X. *et al.*, *Cell*, **84**, 611-622,1996); poor development of mammary gland is observed upon disruption of the gene encoding a LAR enzyme domain (Schaapveld, R. Q. *et al.*, *Dev. Biol.*, **188**, 134-146, 1997); secretion of apolipoprotein B is decreased by suppression of the LAR activity (Phung, T. L. *et al.*, *Biochemical and Biophysical Research Communications*, **237** (2), 367-371, 1997); and loss of expression of LAR diminishes the size of cholinergic nerve cells of prosencephalon basement, thus control by the cholinergic nerve cells at hippocampal dentate gyrus is deteriorated (Yeo, T. T. *et al.*, *J. Neurosci. Res.*, **47** (3), 348-360, 1997). In such a manner, it has been gradually revealed that LAR is bearing several important roles in a living body. Furthermore at present, the most remarkable researches are directed to the relationships between LAR and insulin receptors (Hashimoto, N. *et al.*, *J. Biol. Chem.*, **267** (20), 13811-13814, 1992).

In 1995, a literature was presented which should be noted, reporting that the LAR tyrosine phosphatase activity is abnormally increased in adipose tissue of an obese person, with such an increase being suggested as a cause of onset of insulin resistance and a risk factor of cardiovascular diseases (Ahmad, F. *et al.*, *J. Clin. Invest.*, **95** (6) 2806-2812, 1995). Several reports followed thereafter illustrating that LAR is closely concerned with insulin receptors (Mooney, R. A. *et al.*, *Biochemical and Biophysical Research Communications*, **235** (3), 709-712, 1997; Orr, S. R. *et al.*, *Biochemical Society Transaction*, **25** (3), 452S, 1997; Ahmad, F. *et al.*, *J. Clin. Investigation*, **100** (2), 449-458, 1997; Ahmad, F. *et al.*, *J. Biol. Chem.*, **272** (1), 448-457, 1997; Norris, K. *et al.*, *Febs Letters*, **415**(3), 243-248, 1997; and Li, P. M. *et al.*, *Cellular Signaling*, **8** (7), 467-473, 1996). Further, on the basis of such information, Ahmad, F. *et al.* recently reported that PTP1B may be a therapeutic target of disease states involving in insulin resistance (Ahmad, F. *et al.*, *Metabolism*,

Clinical and Experimental, **46** (10), 1140-1145, 1997).

Next, CD45 among the PTPs, which is also called as leukocyte common antigen (LCA), is a cell surface antigen that is expressed on all hemocytes (leukocytes) other than mature erythrocytes and platelets, and precursor cells thereof. CD45 is a receptor type PTP of which molecular weight ranging between 180 and 220 kDa, having two enzymatic regions intracellularly, and 8 to 9 kinds of isoforms exist which may result from alternative splicing of 3 to 4 exons proximate to N-terminal end of an extracellular region (Saga, Y. *et al.*, *Proc. Natl. Acad. Sci. USA*, **84**, 5364-5368, 1987; Thomas, M. L. *et al.*, *Proc. Natl. Acad. Sci. USA*, **84**, 5360-5363, 1987; and Trowbridge, I. S. *et al.*, *Annu. Rev. Immunol.*, **12**, 85-116, 1994). The amino acid sequences encoded by these exons that may be spliced are rich in serine, threonine and proline, and hardly forms the integrated three-dimensional structures based on α-helix, β-structures and the like, in addition, many O-glycosylated sites are included therein (Barclay, A. N. *et al.*, *EMBO J.*, **6**, 1259-1267, 1987). Therefore, CD45 may be characterized in that the structure of extracellular region can be greatly changed due to changes in the isoforms. Additionally, CD45 is highly expressed in lymphocytes, and intrinsic isomers may be expressed reversibly depending upon cell species, or states of activation of the cells (Thomas, M. L. *et al.*, *Annu. Rev. Immunol.*, **7**, 339-369, 1989; Charbonneau, H. *et al.*, *Annu. Rev. Cell. Biol.*, **8**, 402-493, 1992; and Trowbridge, I. S. *et al.*, *Annu. Rev. Immunol.*, **12**, 85-116, 1994). Furthermore, the sequence of a region, which is interposed between the alternative structures and a transmembrane region, comprises a lot of cysteine, and forms a stabilized structure through disulfide bonds (Thomas, M. L. *et al.*, *Cell*, **41**, 83-93, 1985; Trowbridge, I. S. *et al.*, *J. Biol. Chem.*, **266**, 23517-23520, 1991; Trowbridge, I. S. *et al.*, *Biochim. Biophys. Acta*, **1095**, 46-56, 1991).

It is known that immunogen-specific responsiveness that was intrinsically possessed in the cells is remarkably lowered in variant T cell line with CD45 expression

being lost, thus it is suggested that CD45 may be extremely important in activation of T cell via T cell receptors (TCR), and in functional expression thereof (Charbonneau, H. *et al.*, *Annu. Rev. Immunol.*, 7, 339-369, 1989; Pingel, J. T. *et al.*, *Cell*, **58**, 1055-1065, 1989; Trowbridge, I. *et al.*, *Annu. Rev. Immunol.*, **12**, 85-116, 1994; Koretzky, G. A. *et al.*, *Nature*, **346**, 66-68, 1990; Koretzky, G. A. *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**, 2037-2041, 1991; and Weaver, C. T. *et al.*, *Mol. Cell Biol.*, **11**, 4415-4422, 1991). In addition, it is also suggested that CD45 may participate in activation of Lck ($p56^{lck}$) and Fyn ($p56^{fynT}$), which are tyrosine kinases (PTK) in Src family and are binding to intracellular domain of CD4 and CD8 that are co-receptors of CD45 (Trowbridge, I. S. *et al.*, *Annu. Rev. Immunol.*, 12, 85-116, 1994; and Penninger, J. M. *et al.*, *Immunol. Rev.*, **135**, 183-214, 1993). CD45 dephosphorylates tyrosine residues at a negative regulatory site located in C-terminal end of Lck or Fyn, and consequently, it is believed that Lck or Fyn is autophosphorylated to yield its active form, thus resulting in signal transduction (Penninger, J. M. *et al.*, *Immuno. Rev.*, **135**, 183-214, 1993; Ledbetter, J. A. *et al.*, *Curr. Opin. Immunol.*, **5**, 334-340, 1993; Janeway, C. A. Jr., *Annu. Rev. Immunol.*, **10**, 645-674, 1992; Cahir, McFarland, E. D. *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**, 1402-1406, 1993; Hurley, T. R. *et al.*, *Mol. Cell Biol.*, **13**, 1651-1656, 1993; Sieh, M. *et al.*, *EMBO J.*, **12**, 315-321, 1993; Weiss, A. *et al.*, *Cell*, **76**, 263-274, 1994; and Chan, A. C. *et al.*, *Annu. Rev. Immunol.*, **12**, 555-592, 1994). From the experiments using CD45-deficient cell strain among insulin responsive myeloma cells, it was reported that autophosphorylation of insulin receptors, tyrosine phosphorylation of IRS-1, and activation of PI3 kinase and activation of MAP kinase upon insulin stimulation were all enhanced to three fold in comparison with the strain expressing CD45 (Kulas, D. T. *et al.*, *J. Biol. Chem.*, **271**, 755-760, 1996), therefore, CD45 is supposed to be a negative regulatory factor of insulin similarly to LAR. Moreover, responsiveness of CD45-deficient cells was demonstrated to be recovered upon expression of molecules having only an intracellular region of CD45 by the following experiments. Namely, it was

elucidated that: an enzymatic activity of tyrosine phosphatase can be sufficiently observed even when only the intracellular region of CD45 was expressed in bacterial or baculoviral systems (Ostergaard, H. L. *et al.*, *Proc. Natl. Acad. Sci. USA*, **86**, 8959-8963, 1989; Streuli, M. *et al.*, *Proc. Natl. Acad. Sci. USA*, **86**, 8698-8702, 1989); and signaling madiated by antigen receptors can be recovered by simply transfecting an intracellular region of CD45 to a T cell clone that is negative in CD45 (Volarevic, S. *et al.*, *Science*, **260**, 541-544, 1993; Hovis, R. R. *et al.*, *Science*, **260**, 544-546, 1993; and Desai, D. M. *et al.*, *Cell*, **73**, 541-554, 1993).

Besides, also in cases of B cell, it was indicated that not only early stage of signal transduction, but also ultimate proliferation, or the processes leading to apoptosis may be regulated through CD45 expression, according to the experiments using plasma cell species without expressing CD45 (Justement, L, B. *et al.*, *Science*, 252, 1839-1842, 1991), or the experiments using CD45-negative clone that was established from immature B cell strain (Ogimoto, M. *et al.*, *Int. Immunol.*, 6, 647-654, 1994). These results suggest that CD45 is a molecule that is bearing essential roles in antigen receptors- mediated signal transduction.

Accordingly, from the researches to date on PTPs such as LAR, CD45 and the like, it has been elucidated that PTPs bear extremely important roles in an intracellular signaling system, in conjunction with PTKs.

In 1992, Streuli *et al.* reported that binding between LAR E-subunit and P-subunit may be dissociated due to the noncovalency of their binding, and thus E-subunit is specifically shed from the cell membrane surface (Streuli, M. *et al.*, *EMBO J.*, **11** (3), 897-907, 1992). However, because many researchers have focused the studies using polyclonal or monoclonal antibodies elicited against a LAR E-subunit that is an extracellular domain thereof, a P-subunit even solely having phosphatase activities has been ignored. For example, when an anti-LAR antibody is used intending measurement of activity of LAR phosphatase, total phosphatase activity cannot be measured unless an

antibody to the P-subunit is employed. Further, there exist several isoforms in extracellular domains of LAR family resulting from different types of mRNA splicing (Krueger, N. X. *et al.*, *Cell*, **84**, 611-622, 1996; Mizuno, K. *et al.*, *Mol. Cell Biol.*, **13**, 5513-5523, 1993; and Ogata, M. *et al.*, *J. Immunol.*, **153**, 4478-4487, 1994), thus different specificity to each isoform may be achieved when an antibody to an extracellular domain is used. In view of such circumstances, the present inventors started to produce antibodies to intracellular domains of PTPs.

Meanwhile, anti-CD45 antibodies were conventionally discriminated to antibodies that exhibit specificity to any of the CD45 isoforms having different molecular weights such as T200 or B220, and the antibodies that exhibit specificity only to a particular and restricted isoform, where the latter antibodies have been designated as CD45R (restricted) antibody (McMichael, A. J., In *Leucocyte Typing III*. Oxford University Press, Oxford, 1987). However, as diversities of structure of the extracellular domain of CD45 have been clarified, needs for classification of the specificity of the CD45R antibodies have been raised. Streuli *et al.* classified known human CD45 antibodies by a method using cDNA transfectants, and proposed to refer to the antibody that recognizes the structure that depends on alternative exon 4, 5, or 6 as CD45RA, CD45RB or CD45RC respectively (Streuli, M. *et al.*, *J. Immunol.*, 141, 3910-3914, 1988). Using a similar method, Johnson *et al.* also reported classification of mouse CD45 antibodies (Johnson, P. *et al.*, *J. Exp. Med.*, 169, 1179-1184, 1989).

For further references, known antibodies to PTPs may include an antibody that was generated using 196 amino acid residues as an antigen spanning from the transmembrane (TM) region of CD45 to a part of phosphatase domain 1 (Transduction Laboratories), and an antibody to phosphatase domain 1 (260 amino acid residues) of PTPα (Transduction Laboratories). However, it is unclear how these antibodies are immunospecific to phosphatase domains of LAR, and the other PTPs.

SUMMARY OF THE INVENTION

One aspect of the present invention is to provide an antibody having specificity to intracellular domains of two or more kinds of PTPs, and in particular, an antibody having specificity to an intracellular domain of at least one or more receptor type PTP. Particularly, the antibody according to the present invention may be an antibody having specificity to intracellular domains of LAR and/or CD45, preferably both of the intracellular domains of LAR and CD45. The antibody may preferably have specificity to phosphatase domains of PTPs.

The above-described antibody may be preferably generated using a polypeptide corresponding to a P-subunit of LAR that is encoded by a nucleotide sequence set out in SEQ ID NO: 1 or any of fragments thereof as an antigen. Further, preferred antibody may be a monoclonal antibody because of its immunospecific property.

In addition, the antibody may be generated using a fusion protein comprising a protein tyrosine phosphatase domain and another protein or a polypeptide, as an immunogen. The protein tyrosine phosphatase domain that is a member of the fusion protein may preferably be a phosphatase domain of LAR, and as the another protein or the polypeptide, GST (glutathione-S-transferase) may be particularly suitable, besides, polyhistidine (preferably 6 histidine residues), calmodulin binding peptide (CBP), protein A and the like may be employed as the another protein or the polypeptide.

When polyhistidine is employed, absorption to nickel-chelating resin can be utilized for isolation and purification of the fusion protein expressed by a gene recombinant process, wherein addition of EDTA or imidazole substance as well as pH change may be adopted for dissociating the protein from the resin. When CBP is employed, the expressed fusion protein may be subjected to an affinity chromatography using calmodulin affinity resin, and then may be dissociated from the resin by adding

EGTA. In addition, when protein A is employed, the expressed fusion protein may be subjected to an affinity chromatography using IgG sepharose (e.g., IgG Sepharose 6FF), and then may be dissociated from the resin by changing pH.

Moreover, another candidate for the protein or the polypeptide fragment to be employed in the fusion protein may include for example, Xpress, Thioredoxin, c-myc, V5, HA/c-myc and the like. For isolation and purification of the intended fusion protein with a LAR phosphatase domain, expression of the protein may be followed by subjecting to an antigen-antibody affinity column.

The aforementioned preferable immunogen of the present invention, namely a fusion protein of GST and a LAR phosphatase domain, may be suitably produced by: culturing *Escherichia coli* transformed or transfected with an expression vector comprising a coding region of GST gene and a coding region of a phosphatase domain of LAR gene at 20-30°C for 16-24 hours, preferably at 23-25°C for 18 hours; and then isolating the fusion protein from the culture fluid and/or bacterial cells. Thus obtained fusion protein may be further purified based on an affinity to a support carrying glutathione, e.g., glutathione sepharose beads, wherein the elution of the fusion protein from the glutathione sepharose beads may be performed by boiling in the presence of a detergent. The detergent may include sodium dodecyl sulfate, CHAPS (3-[(3-cholamide propyl) dimethylammonio]-1-puropane sulfonate), deoxycholic acid, digitonin, n-dodecylmaltoside (1-O-n-dodecyl-β-D-glucopyranosyl (1-4) α-D-glucopyranoside), Nonidet™ P40 (ethylphenolpoly (ethylene glycol ether)n), n-octylglucoside (1-O-n-octyl-β-D-glucopyranoside), sucrose monolaurate, Tesit™ (dodecylpoly (ethylene glycol ether)n), Triton™ X-100 (octylphenolpoly (ethylene glycol ether)n), Tween™ 20 (poly (oxyethylene) n-sorbitan-monolaurate), N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and the like [any of 'n' represents an integer number which is more than or equal to 1]. When elution of the

fusion protein is carried out, the resin may be boiled at 100°C for 5-10 minutes in the presence of such detergents at a concentration that does not lead any problems to an animal to be administered, preferably in the presence 0.1% of sodium dodecylsulfate. Accordingly, a purified fusion protein, which is preferable as a contemplated immunogen, can be obtained.

When a monoclonal antibody is generated using such a fusion protein as an immunogen, a protein tyrosine phosphatase domain, preferably a LAR phosphatase domain may be employed for screening the antibody, however, it is more preferable to perform the screening using the fusion protein as an immunogen in terms of the specificity.

The monoclonal antibody of the present invention may include a monoclonal antibody that is produced from mouse/mouse hybridoma cells, and has specificity to intracellular domains of phosphatase subunits of LAR and CD45. For example, such an antibody of the present invention may include a monoclonal antibody having an apparent molecular weight of about 146 kDa on SDS-PAGE. The antibody can be applied as a tool for further elucidation of the mechanisms of an insulin signaling system, for developing useful diagnostic methods of insulin resistance and NIDDM, and for prophylaxis, therapeutics and diagnosis of several kinds of pathological states relating to syndrome X based on insulin resistance.

Further aspect of the present invention is to provide a hybridoma cell line producing the above-mentioned monoclosnal antibody. Such a hybridoma cell line may include mouse/mouse hybridoma cell line YU2, which was deposited on May 7, 1998, with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-1-320, Higashi, Tsukuba, Ibaraki, JAPAN, and assigned Accession No. FERM BP-6344.

The antibody of the present invention has specific immunoreactivity with PTP protein, and fragments and polypeptides that comprise at least a part (more than or equal to

three amino acid residues, preferably, more than or equal to five amino acid residues) of an intracellular domain of PTP (the fragment and polypeptide are hereinafter collectively referred to as 'PTP derived molecules'), which was derived from natural sources, or wholly or partially synthesized (such as those chemically synthesized, or recombinantly synthesized).

Another aspect of the present invention is to provide a method for generating an antibody having specificity to two or more kinds of protein tyrosine phosphatase subunits, wherein the aforementioned fusion protein comprising a protein tyrosine phosphatase domain and another protein or a polypeptide fragment, preferably a GST-LAR phosphatase domain fusion protein, is employed as an immunogen. In this aspect of the present invention, the available another protein or a polypeptide fragment except GST to be a member of the fusion protein, and purification process of the fusion protein are as set forth above.

Further, a fusion protein comprising GST and a LAR phosphatase domain which is a preferable immunogen may be suitably produced by: culturing *Escherichia coli* transformed or transfected with an expression vector comprising a coding region of GST gene and a coding region of a phosphatase domain of LAR gene at 20-30°C for 16-24 hours, preferably at 23-25°C for 18 hours; and then isolating the fusion protein from the culture fluid and/or bacterial cells. Thus obtained fusion protein may also be further purified based on an affinity to a support carrying glutathione, e.g., glutathione sepharose beads, wherein the elution of the fusion protein from the glutathione sepharose beads may be performed by boiling in the presence of a detergent, as set forth above, and again, for eluting the fusion protein, the resin may be boiled at 100°C for 5-10 minutes in the presence of the detergent at a concentration which does not lead any problems to an animal to be administered, preferably in the presence of 0.1% of sodium dodecylsulfate. Accordingly, the purified fusion protein, which is preferable as a

contemplated immunogen, can be obtained.

In a method of generating a monoclonal antibody by using such a fusion protein as an immunogen, a protein tyrosine phosphatase domain, preferably a LAR phosphatase domain may be employed for screening the antibody, however, it is more preferable to perform the screening using the fusion protein that was employed as an immunogen in terms of the specificity.

Furthermore, the present invention provides a method of isolating a novel PTP comprising a step for screening PTP, wherein the aforementioned antibody is used in the step of screening. It is contemplated that expression screening of a cDNA library may be performed in the screening step.

In yet another aspect of the present invention, a method of quantitative determination of PTP and/or PTP derived molecules is provided. In this method, an amount of PTP protein and/or a fragment or a polypeptide that comprises at least a part of a PTP intracellular domain, which is contained in a test sample, is determined using the antibody set forth above. The antibody set forth above is used preferably in any of immunoblotting, immunoprecipitation and ELISA, for determination in this method.

Still another aspect of the present invention is to provide a method for quantitative determination of PTP protein and/or PTP derived molecules comprising the steps of: isolating PTP and/or a fragment or a polypeptide that comprises at least a PTP intracellular domain from a test sample using the antibody set forth above; and measuring an activity of the isolated PTP and/or PTP derived molecules. In this step for isolation, affinity chromatography and/or immunoprecipitation by using a support that was bound with the aforementioned antibody may be suitably utilized.

In yet another aspect of the present invention, a method for producing PTP and/or PTP derived molecules is provided, comprising the step of isolating PTP protein and/or a fragment or a polypeptide that comprises at least a PTP intracellular domain using the

antibody set forth above. In this step for isolation, affinity chromatography and/or immunoprecipitation by using a support that was previously bound with the antibody described above may be suitably utilized.

Further aspect contemplated by the present invention is to provide a method for identifying the presence of PTP and/or PTP derived molecules within tissue comprising the step of performing immunohistological examination using the aforementioned antibody. As the immunohistological examination, the technique such as *in situ* immunohistological staining with a labelled antibody may be adopted, thus PTP protein and/or a fragment or a polypeptide that comprises at least a PTP intracellular domain, can be detected.

Besides, the present inventors found that a monoclonal antibody having specific immunoreactivity with LAR can specifically recognize thyroid carcinoma cells. Therefore, the above-mentioned antibody of the present invention is presumed to be useful in diagnosis, therapy and the like of thyroid cancer.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1A-B. Figure 1A Figure 1A

~~Figure 1~~ is a schematic drawing depicting a subunit structure of LAR (~~a~~); and a schematic drawing illustrating the mutated LAR phosphatase domain structures inside the membrane (~~b~~) prepared as demonstrated in Experimental Examples. Figure 1B

Figure 2 represents immunoblots illustrating time dependent tyrosine phosphorylation induced by insulin stimulation in COS cells that were cotransfected with LAR/CS and wild type insulin receptor (IR).

Figure 3 represents immunoblots illustrating phosphorylation-dephosphorylation in COS cells that were cotransfected with wild type or mutants of LAR, and wild type insulin receptor.

Figure 4 represents an immunoblot illustrating dephosphorylation of a β-chain of insulin receptor by wild type or mutants of LAR.

Figure 5 represents an immunoblot illustrating tyrosine phosphorylation in COS cells that were cotransfected with wild type or mutant of insulin receptor, and LAR/CS.

Figure 6 represents SDS-polyacrylamide gel, showing a molecular weight of the antibody YU2 of the present invention.

Figures 7A-B
~~Figure 7~~ represents immunoblots showing immunospecificity of the antibody YU2 of the present invention.

Figure 8 represents analytical results of immunoblotting on CD45 using the antibody YU2 of the present invention

~~Figure 9 represents homology of amino acid sequences of intracellular domains~~ of LAR and CD45.

Figure 10 is a schematic drawing depicting a signal transduction cascade of insulin that is controlled by phosphorylation-dephosphorylation in which insulin receptor and LAR participate.

BEST EMBODIMENT FOR CARRYING OUT THE INVENTION

Experimental Example 1: Tyrosine phosphorylation of insulin receptors by LAR mutants and studies on association between LAR and insulin receptors

First, in order to elucidate the mechanisms controlling signal transduction of insulin by LAR, analysis was performed with a strategy in which mutated LAR is used that was prepared by substitution of cysteine with serine, which exists in a catalytic center of PTP domain of LAR.

A) Expression vector of LAR and insulin receptors

Three kinds of LAR expression vectors were used, i.e., (a) LAR WT: human wild type LAR (SEQ ID NO: 3); (b) LAR C/S: mutated LAR, having substitution of cysteine in a catalytic center of LAR-PTP domain 1 (amino acid residue position 1552 of SEQ ID NO: 3) for serine by substituting nucleotide G, position 4983 of SEQ ID NO: 3, with C; and (c)

LAR DC/S: further mutated one in addition to LAR C/S, with substitution of cysteine in LAR-PTP domain 2 (amino acid residue position 1813 of SEQ ID NO: 3) for serine by substituting nucleotide G, position 5856 of SEQ ID NO: 3, with C (see, Fig. 1 (b)), each of which was incorporated into pMT expression vector (see, Streuli M. *et al.*, *EMBO J.*, **11**, 897-907, 1992; and Streuli M. *et al.*, *EMBO J.*, **9**, 2399-2407, 1990).

Meanwhile, the employed insulin receptor expression vectors were: (a) IR WT: wild type; and (b) IR K1018M: mutated insulin receptor having substitution of lysine, position 1018 of ATP binding site of wild type insulin receptor, with methionine resulting in deficiency of tyrosine kinase activity, each of which cDNA was incorporated to downstream of SRα promoter (see, Kanai F. *et al.*, *Biochemical and Biophysical Research Communications*, **195**, 762-768, 1993).

B) Transfection into COS-7 cells

COS-7 cells were seeded into RPMI 1640 medium (Nissui Pharmaceutical Co., LTD.) supplemented with 10% fetal calf serum at 1.0×10^6 cells/8 mL/90 ϕ dish, then after 16 hours incubation, expression vectors of LAR C/S and IR WT were cotransfected into COS-7 cells using DEAE-dextran method. The LAR C/S employed was a vector that was revealed to include complete deficiency in tyrosine phosphatase activities *in vitro* (Streuli M. *et al.*, *EMBO J.*, **9**, 2399-2407, 1990) according to mutation as mentioned above in paragraph A, (b).

Cotransfection was performed according to the following procedure. Initially, 40 µl of 10 mM chloroquine was added to 4 ml of RPMI 1640 medium (10.2 g/L of RPMI 1640 (Nissui Pharmaceutical Co., LTD.) containing 0.3 g of glutamine and 0.1 g of kanamycin, pH 7.4 that was adjusted with 10% $NaHCO_3$) containing 2% FCS. To 2 ml of this solution, 5 µg of LAR expression vector and 1 µg of IR expression vector were added, on the other hand, 16 µl of 100 mg/ml DEAE-dextran was added to the remaining 2 ml of the solution. Then, both solutions were mixed thoroughly with stirring. Thus prepared

solution of expression vector, 3.75 ml was plated at 1.0 x 10^6 cells/8 ml/dish, and was added to COS-7 cells that had been precultured for 16 hours at 37°C, in a 5% CO_2 incubator. Following 4 hours culture under the similar conditions to the preculture, the cells were treated with 10% DMSO solution for 2 minutes, then washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 12H_2O$, 1.4 mM KH_2PO_4), thereafter, 8 ml of RPMI1640 containing 10% FCS was added thereto, and cultured for 48 hours at 37°C in an incubator that was adjusted to 5% CO_2.

C) Insulin stimulation and preparation of cell lysate

COS-7 cells after completing the transfection were incubated for 16 hours in serum free RPMI 1640 culture medium, followed by stimulation with 10^{-7} M insulin (Seikagaku Corporation) for determined periods, i.e., 0, 1, 5, 15 and 30 minutes. Stimulation for 0 minute was conducted by standing on ice without incubating at 37°C, although insulin was added similarly. After each of the time elapsed from the beginning of insulin stimulation, culture fluid was entirely aspirated therefrom, and 5 ml of PBS w/Inh. (PBS containing tyrosine phosphatase inhibitors: 1 mM sodium vanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 5 mM EDTA-2Na, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 12H_2O$, 1.4 mM KH_2PO_4) was immediately added.

Following washes of the whole cells with PBS w/Inh., the fluid was removed by aspiration, and 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM iodoacetamide, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.4 mM sodium vanadate, 0.1 mM oxidized phenylarsine, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) was added to the cells, which were thereafter collected with a cell scraper. The cell suspension was transferred to a 1.5 ml tube, and then incubated at 4°C for 30 minutes to effect complete lysis of the cells. Supernatant, which was obtained by centrifuge of the fluid at 12,000 rpm, 4°C for 10 minutes following incubation was employed as a cell lysate in the experiments set forth

below.

D) Immunoprecipitation

Immunoprecipitation was performed for the cell lysate obtained as above paragraph C, with an anti-LAR E-subunit antibody (a mixture of 7.5 μg of 11.1A and 7.5 μg of 75.3A, see, Streuli M. *et al.*, *EMBO J.*, **11**, 897-907, 1992). To 1 ml of the above cell lysis solution, 15 μg of MOPC 21 (mouse IgG1κ: Sigma Corporation) as a mock was added, then the solution was incubated at 4°C for one hour, added with 20 μl of γ-bind (GammaBind Plus Sepharose: Pharmacia Biotech Inc.) thereto, and further incubated for one hour at 4°C to execute preabsorption. The solution was centrifuged at 4°C, 12,000 rpm for 10 minutes, then 950 μl of the supernatant was transferred to another tube. Next, 15 μg of anti-LAR E-subunit antibody was added to the supernatant, then the solution was incubated at 4°C for one hour, added with 20 μl of γ-bind thereto, and further incubated for one hour at 4°C. After centrifuge at 4°C, 12,000 rpm for 10 minutes, the precipitate was washed with 1 ml of lysis buffer twice, then once with PBS w/Inh., and suspended in 20 μl of SDS sample buffer. The suspension was heated for 5 minutes in a boiling water bath to prepare a sample for electrophoresis.

E) Immunoblotting

The above-mentioned sample was subjected to electrophoresis using 7.5% SDS-polyacrylamide gel, followed by transfer to a nitrocellulose membrane (Schleicher & Schuell) using a transfer device at 400 mA for 4 hours. Then blocking was conducted by incubating the membrane in 3% bovine serum albumin solution for longer than 30 minutes at a room temperature. After washing with sufficient volume of TBS-T (TBS with Tween 20: 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20) for 10 minutes more than twice, an anti-phosphotyrosine antibody (4G10, UBI) that was 50,000-fold diluted with TBS-T, the anti-LAR E-subunit antibody or an anti-insulin receptor β-chain antibody (UBI) was added thereto, then the mixture was shaken for one hour at a room

temperature. After washing with sufficient volume of TBS-T for 5 minutes more than three times, 15 ml of TBS-T solution containing HRP-labelled anti-mouse IgG antibody (horseradish peroxidase-labelled anti-mouse IgG: Santa Cruz Biotechnology, Inc.) 1.5 ml was added thereto, and shaken for one hour at a room temperature. After washing with sufficient volume of TBS-T for 5 minutes more than three times, bands of the protein were detected that can bind to each of the antibodies, by means of a chemiluminescence method using a kit of luminescence reagents (Wako Pure Chemical Industries, Ltd.).

F) Results

As results of immunoblotting with the anti-phosphotyrosine antibody following to immunoprecipitation with the anti-LAR E-subunit antibody of cell lysate prepared after stimulation with insulin for determined time periods of cotransfected COS-7 cells with LAR C/S and IR WT in the above-described manner, tyrosine phosphorylation of an insulin receptor β-chain as well as a 85 kDa protein could be observed with the insulin stimulation for 1 minute. Such tyrosine phosphorylation could also be successively observed with the insulin stimulation for 30 minutes (see, Fig. 2A).

Furthermore, results from the immunoblotting with the anti-LAR E-subunit antibody (Fig. 2B), the anti-insulin receptor β-chain antibody (Fig. 2C) and the anti-phosphotyrosine antibody (Fig. 2A) demonstrated that LAR and insulin receptor may associate depending on the presence or absence of tyrosine phosphorylation of the insulin receptor.

Experimental Example 2: Studies on tyrosine dephosphorylation of insulin receptor by various LAR (1)

Next, COS-7 cells were similarly cotransfected with LAR WT, LAR C/S or LAR DC/S, and IR WT followed by stimulation with insulin for 5 minutes, and immunoprecipitation with the anti-LAR E-subunit antibody, and then immunoblotting with

various types of antibodies for the precipitates was carried out. Consequently, tyrosine phosphorylation of the insulin receptor β-chain or the 85 kDa protein could not be detected for the cells cotransfected with insulin receptor and LAR WT, in comparison with the cells cotransfected with LAR C/S or LAR DC/S (see, Fig. 3A).

Additionally in these experiments, amounts of expression of LAR (Fig. 3C) and the insulin receptor (Fig. 3D) were almost identical in both of the cotransfectants, therefore LAR WT was suggested to dephosphorylate the phosphorylated tyrosine of the insulin receptor β-chain as well as the 85 kDa protein.

Further, when the immunoprecipitates with the anti-LAR E-subunit antibody were immunoblotted using the anti-insulin receptor β-chain antibody, the cotransfectant with LAR DC/S showed a weaker band of an insulin receptor β-chain, compared to the cotransfectant with LAR WT or LAR C/S (Fig. 3B).

These results indicate that the association between insulin receptor and LAR DC/S is weaker, when compared with that of LAR WT or LAR C/S. The only one difference between LAR C/S and LAR DC/S is one amino acid residue position 1813 of phosphatase domain 2, accordingly, this domain 2, which was postulated to involve in binding with substrates without tyrosine phosphatase activity, was proved to be playing a role in binding between LAR and insulin receptor.

Experimental Example 3: Studies on tyrosine dephosphorylation of insulin receptor by various LAR (2)

In order to further study as to whether tyrosine dephosphorylation of insulin receptor occurs only in cases where LAR was bound, or in every insulin receptor, cell lysate of the cotransfectant was subjected to electrophoresis, and then immunoblotted with the anti-phosphotyrosine antibody. Consequently, tyrosine dephosphorylation of insulin receptor was markedly found only in cells that had been cotransfected with LAR WT (see,

Fig. 4).

Experimental Example 4: Studies on tyrosine phosphorylation of insulin receptor in the presence of LAR C/S

In order to elucidate whether tyrosine phosphorylation of the 85 kDa protein is effected by a tyrosine kinase activity of insulin receptor, COS-7 cells were cotransfected with LAR C/S, and IR WT or IR K1018M(IR MT) having a deficiency in tyrosine kinase of insulin receptor. Following insulin stimulation of the cells for 5 minutes, immunoprecipitation was performed with the anti-LAR E-subunit antibody, and immunoblotting with the anti-phosphotyrosine antibody was carried out (see, Fig. 5). Consequently, the cells cotransfected with IR WT showed tyrosine phosphorylation of an insulin receptor β-chain and the 85 kDa protein upon stimulation with insulin, however, the cells cotransfected with IR K1018M showed no such phosphorylation at all.

From these results, it was revealed that rapid tyrosine phosphorylation of insulin receptor β-chain occurs upon binding of insulin to insulin receptor; and that the insulin receptor tyrosine kinase leads tyrosine phosphorylation of the 85 kDa protein.

The 85 kDa protein was therefore speculated as a P-subunit of LAR of which binding to insulin receptor was demonstrated.

Example 1: Generation of anti-tyrosine phosphatase P-subunit antibodies

Anti-tyrosine phosphatase P-subunit antibodies were generated according to the following procedures.

A) Preparation of immunogen

Glutathione-S-transferase-LAR fusion protein (GST-LAR) was employed as an immunogen. *E.coli* AD202 was transformed with an expression vector, pGEX-2T vector (Pharmacia Biotech Inc.), which was incorporated to its *Bam*HI/*Eco*RI site with cDNA

corresponding to 607 amino acids spanning from the end of a transmembrane region of a LAR P-subunit to the entire cytoplasmic region (SEQ ID NO: 1, 3467 bp) according to a general procedure. After the *E.coli* was incubated overnight in LB (Amp. +) agar medium (LB (Amp. +) described below containing 7.5 g of agar), single colony was inoculated to 50 ml of LB (Amp. +) medium (containing triptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, 5 N NaOH 0.2 ml/L, and ampicillin 50 μg / ml), and further incubated overnight. Then the incubation mixture of *E.coli* was inoculated to 500 ml of LB (Amp. +) medium, and incubated at 37°C until absorbance at 600 nm reaches to approximately 1.0, followed by addition of 50 μl of 1 M IPTG (isopropyl-β-D(-)-thiogalactopyranoside, Wako Pure Chemical Industries, Ltd.) and an incubation at 25°C overnight. Thus resulted culture was centrifuged at 3,000 rpm, 4°C for 15 minutes, and the precipitated bacterial bodies were suspended in 50 ml of NETN (0.5 % Nonidet P-40 , 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 100 mM NaCl). Thereafter, the suspension was subjected to twice repeated treatments of ultrasonication for 1 minute and standing on ice for one minute, and then centrifuged at 14,000 rpm, 4°C for 20 minutes to obtain the supernatant. To 10 ml of the lysate of the *E.coli*, 100 μl of suspension of glutathione sepharose beads (Glutathione Sepharose 4B (Pharmacia Biotech Inc.) that had been prepared by washing three times, and suspended in 50% NETN) was added, and then incubated for 30 minutes at a room temperature. Thus resulted suspension was centrifuged at 3,000 rpm, 4°C for 5 minutes, and supernatant was removed. The precipitated glutathione sepharose beads were washed twice with NETN, then once with PBS, thereafter 100 μl of SDS sample buffer (125 mM Tris-HCl pH 6.8, 0.1% sodium dodecylsulfate, 5% 2-mercaptoethanol) was added thereto, and heated in a boiling water bath for 10 minutes to elute the GST-LAR fusion protein. The eluate from which the beads were eliminated was concentrated by centrifuge using Centricon-10 (Amicon) at 3,000 rpm, 4°C for 45 minutes. One ml of PBS was added to the concentrate in order to bufferize the solution, and the solution was

concentrated again by centrifuge at 3,000 rpm, 4°C for 45 minutes. This process for bufferization was repeated twice, and thus resulted solution was employed as an immunogen solution. Purification and concentration of the antigenic protein were confirmed by SDS-polyacrylamide gel electrophoresis.

Meanwhile, on a final immunization, the antigen solution was prepared in a different process because it should be administered intravenously. The lysate of the above-described *E. coli* that is expressing GST-LAR fusion protein was incubated with glutathione sepharose beads, and after centrifuge, the precipitated beads were washed twice with NETN, and three times with PBS. Next, 100 µl of GSH elution buffer (20 mM glutathione, 1M Tris-HCl, pH 9.6) was added thereto, and the mixture was gently stirred for 10 minutes at a room temperature to accomplish the elution of GST-LAR. After repeating the steps of centrifuge at 3,000 rpm, 4°C for 5 minutes and recovering the supernatant three times in total, the total eluate was dialyzed in saline at 4°C for 2 days, then thus obtained solution was employed as an immunogen solution for intravenous administration.

B) Immunization

Eight female Balb/c mice of 6 weeks old received intraperitoneal administration of pristane (2,6,10,14-tetramethylpentadecane, Sigma Corporation) at 0.5 ml/animal. After 2 weeks passed, the antigen solution for intraperitoneal immunization that was emulsified by blending with Freund's complete adjuvant (Gibco Inc.) at a ratio of 1:1 was intraperitoneally administered at about 10 µg of GST-LAR fusion protein per one mouse. Thereafter, the antigen solution for intraperitoneal immunization that was admixed with Freund's incomplete adjuvant (Gibco Inc.) at a ratio of 1:1 was prepared to be about 30-70 µg of GST-LAR per one mouse, and the mixture was intraperitoneally administered four times approximately once every 2 weeks. On day 4 after the fourth immunization, blood was collected from ocular fundus vein, and an antibody titer in the serum was determined by ELISA method.

C) ELISA

Protein solutions of GST-LAR and GST alone that were prepared similarly to the procedure of preparation of the antigen for intravenous immunization were respectively dialyzed against purified water at 4°C overnight. These solutions were adjusted to 0.5 μg / ml in PBS, and subjected to absorption to an ELISA plate (Falcon 3911 MicroTest ™ Flexible Assay Plate) at 50 μl/well for one hour. After five times washes with wash buffer (PBS containing 0.05% Tween20), blocking with 5% skim milk (prepared by dissolving 2.5 g of skim milk in 50 ml of PBS) was conducted. Following washes, the serum as obtained in the above section B was diluted to 16,000 fold with dilution buffer (PBS containing 0.25% BSA), and was added to the wells at 50 μl/well, and then incubated for one hour in a wet box. After washing the plate, HRP-labelled anti-mouse IgG antibody that was diluted to 1,000 fold was added to the plate at 50 μl/well, and incubated for one hour. Following washes with wash buffer four times and once with PBS, a substrate solution of *o*-phenylenediamine (Wako Pure Chemical Industries, Ltd.) that was dissolved in a citrate buffer (prepared by dissolving 5.6325 g of citric acid monohydrate and 18.35 g of $Na_2HPO_4 \cdot 12H_2O$ in purified water to make 500 ml in total) at a concentration of 1 mg /ml was added at 50 μl/well, allowed for reaction for 30 minutes, and then 50 μl of 10% H_2SO_4 was added to terminate the reaction. Fifty μl of the solution was transferred to each well of a 96-well plate (Sumitomo Bakelite Co., LTD.) for measurement, and then absorbance at 450 nm was measured.

D) Cell fusion

Two mice that showed elevation of the antibody titer to GST-LAR in accordance with the results of the above ELISA were finally immunized by intravenous administration, and spleen was excised therefrom on the third day to prepare splenocytes according to an ordinary procedure.

Parent cells employed for cell fusion were Balb/c mouse-derived myeloma cell

strain NS1 that was previously selected in a medium containing 20 μg /ml 8-azaguanine, and confirmed as hypoxanthine, guanine, phosphoribosyl transferase (HGPRT) deficient strain. Cell fusion and cloning were performed with 2 x 10^7 of NS1 cells and 1 x 10^8 of splenocytes, using ClonaCell™- HY Hybridoma Cloning Kit (StemCell Technologies Inc.).

Screening of the supernatant from the culture of the cloned hybridoma was carried out according to ELISA method described in section C above, with 50 μl of the supernatant of hybridoma culture using plates that were bound with 0.5 μg/ml protein solution of GST, GST-LAR or GST-CD45 (Furukawa, T. *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**,10928-10932,1994) prepared by the similar method for preparation of the antigen for intravenous immunization as described above. In this ELISA method, hybridoma was selected, which did not show any immune response to the wells bound with GST, but showed an immune response to the wells bound with GST-LAR or GST-CD45. Passage culture of the cloned hybridoma was conducted in RPMI 1640 medium (Nissui Pharmaceutical Co., LTD.) containing 10% fetal bovine serum (Gibco Inc.).

Through screening by ELISA method of the culture supernatant in this manner from the hybridoma that was HAT selected, a clone YU2 having both stable antibody producibility and proliferation ability could be obtained.

This hybridoma cell line YU2 was deposited on May 7, 1998, with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-1-320, Higashi, Tsukuba, Ibaraki, JAPAN, and assigned Accession No. FERM BP-6344.

E) Typing of monoclonal antibody

Supernatant of 0.5 ml from culture of hybridoma YU2 obtained in the above section D was diluted with 4.5 ml of TBS-T, and the isotype was determined for 3 ml of the diluted solution using mouse monoclonal antibody isotyping kit (Amersham International

plc.). As a result, the isotype of the antibody was proved to be IgG1κ.

F) Generation and purification of monoclonal antibody

Balb/c mice of 6 weeks old received intraperitoneal administration of pristane at 0.5 ml/animal, and after 10 days, hybridoma cell YU2 that was obtained by cloning in section D above was intraperitoneally injected at 2.5×10^6 - 1.3×10^7 cells/0.5 ml/animal. Abdominal hypertrophy was observed approximately 10 days thereafter, accordingly, ascites fluid was collected several times using a 20-gauge injection needle. Thus collected ascites fluid was centrifuged at 1,000 rpm, 4 °C for 5 minutes to separate supernatant and precipitate. The supernatant was incubated at 37 °C for 30 minutes, and allowed to stand at 4 °C overnight. Following centrifuge at 12,000 rpm, 4°C for 10 minutes, the monoclonal antibody YU2 was purified using an affinity column HiTrap ProteinG (Pharmacia Biotech Inc.) from the 1.5 ml of supernatant thus obtained. Absorbance at 280 nm of the antibody solution thus obtained was measured, and then concentration of the antibody was calculated using molecular extinction coefficient of mouse IgG.

In addition, a molecular weight of the monoclonal antibody YU2 was estimated from mobility on SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 6. As is clear from the Fig. 6, the monoclonal antibody YU2 comprises H-chain of about 48 kDa and L-chain of about 25 kDa, having a total molecular weight of about 146 kDa.

Example 2: Studies on specificity of monoclonal antibody (1)

An expression vector of LAR WT was transfected into COS-7 cells according to the procedures described in Example 1, sections A and B. Following immunoprecipitation of the cell lysate with the purified monoclonal antibody obtained in Example 1, immunoblotting was carried out. As a control on immunoprecipitation, MOPC 21 was employed because the anti-LAR E-subunit antibody (*supra*), an anti-CD45

antibody (Santa Cruz Biotechnology, Inc., 35-Z6), and the monoclonal antibody YU2 all belong to IgG1 subclass.

On the analyses using the LAR enforced expression system in COS-7 cells, the monoclonal antibody YU2 recognized the protein of 85 kDa that corresponds to a LAR P-subunit and the protein of about 200 kDa that corresponds to a precursor, following immunoprecipitation with the anti-LAR E-subunit antibody (see, Fig. 7B).

Moreover, upon immunoblotting with an antibody that recognizes a LAR E-subunit after immunoprecipitation of cell extract of COS-7 cells transfected with LAR using these antibodies (IgG1, IgG2b, or YU2), detection of the protein of 150 kDa that corresponds to a LAR E-subunit and the protein of about 200 kDa that corresponds to a precursor was restricted only to that immunoprecipitated with the antibody YU2 (see, Fig. 7A).

From the results set forth above, it was revealed that the monoclonal antibody YU2 could be utilized for immunoprecipitation and immunoblotting of a LAR P-subunit.

Meanwhile, YU2 exhibited the reactivity in ELISA using GST-CD45 (Furukawa, T. *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**, 10928-10932, 1994) as an antigen, accordingly, it was speculated that YU2 recognized a common antigen of LAR and CD45 (presumably, a sequence in PTP domain that is conserved in both proteins) as an epitope.

Example 3: Studies on specificity of monoclonal antibody (2)

In order to further investigate specificity of the monoclonal antibody YU2, expression of CD45 in COS-7 cells was enforced according to the similar procedure described in Experimental Example 1, thereafter the cell extract was subjected to immunoblotting analysis using YU2, and the bands were identified at positions corresponding to about 200 kDa and about 180 kDa (see, Fig. 8).

These bands were also detected at the same positions when the blot was reprobed

using commercially available anti-CD45 antibody, accordingly, it was verified that these bands are derived from CD45.

From the results of the above Examples 2 and 3, it was demonstrated that YU2, which was picked up as a clone that exhibits reactivity with both of the intracellular domains of LAR and CD45 in screening of the hybridoma by means of ELISA method, can recognize CD45 also in immunoblotting.

Homology between amino acid sequences of intracellular domains of LAR and CD45 is shown in Fig. 9. In the figure, "•" denotes the identical amino acid, whereas ":" denotes the similar amino acid between both amino acid sequences. When the amino acid sequences from the phosphatase domain 1 to C-terminal end in the intracellular domains of LAR and CD45 are compared, it was revealed that the homology was 39.4%. Among them, 12 amino acids corresponding to the vicinity of consensus sequence that carries tyrosine phosphatase activity in domain 1 (Val-Val-His-Cys-Ser-Ala-Gly-Val-Gly-Arg-Thr-Gly; SEQ ID NO: 4 (amino acids from position 245 to 256 in SEQ ID NO: 1) are completely identical (the portion depicted with outline characters in Fig. 9). It is reported that a polypeptide must include approximately 8 to 10 amino acids in order to be a possible antigen determinant, therefore, it is expected that YU2 may recognize the consensus sequence of the phosphatase comprising the 12 amino acids set out in SEQ ID NO: 4 as an epitope.

In Fig. 9, eight sites corresponding to the portions of consensus sequences in the intracellular domains of LAR and CD45 with other known PTPs (namely, PTPα, β, γ, δ, ε, σ, μ, κ, η, ζ and the like) are depicted with closed boxes. These consensus sequences in the eight sites are repetitive sequences of four kinds of sequences ((1) to (4) in Fig. 9) of which details are as follows:

(1) Phe-Trp-(Arg/Glu/Leu)-Met-(Val/Ile/Cys)-Trp (SEQ ID NO: 5)

(2) Lys-Cys-(Ala/Asp)-(Gln/Glu/Lys)-Tyr-Trp-Pro (SEQ ID NO: 6)

(3) Trp-Pro-Asp-(His/Phe)-Gly-Val (SEQ ID NO: 7)

(4) Pro-Xaa-(Ile/Val)-(Ile/Val)-His-Cys-Xaa-Ala-Gly-Xaa-Gly-Arg

-(Thr/Ser)-Gly (SEQ ID NO: 8)

The above-mentioned identical sequences of LAR and CD45 set out in SEQ ID NO: 4 are included in the consensus sequence (4) in domain 1... It is expected that the antibody of the present invention that can recognize such a consensus sequence of PTP as an epitope can be utilized advantageously in analysis and quantitative determination of PTPs, identification, detection, isolation and purification of novel PTPs.

In addition, as is shown in Figure 10, tyrosine kinase activity is increased upon autophosphorylation of the β-chain of insulin receptor when insulin is bound to the α-chain of the insulin receptor. Due to the activity of the tyrosine kinase, insulin actions such as glucose uptake, glucose metabolism, and cell proliferation may be finally achieved. The present inventors clarified that the activated insulin receptor may change back into the inactivated state through tyrosine dephosphorylation by LAR (see, International Application PCT/JP98/02542, filed on June 5, 1998). Moreover, the possibilities were suggested that: (1) insulin receptor tyrosine kinase may phospholylate tyrosine in intracellular domain of LAR; (2) such phosphorylation may participate in determination of substrate specificity of LAR, or in increase of phosphatase activity; and (3) LAR may control its own enzymatic activity through autodephosphorylation of the phosphorylated tyrosine, accordingly, it was demonstrated at the molecular level that acceleration of the enzymatic activity of LAR may cause insulin resistance. According to the antibody of the present invention, elucidation of signal transduction mechanisms and various other control mechanisms in which phosphorylation and/or dephosphorylation may participate can be achieved, where LAR and CD45 as well as other PTPs may be involved.

INDUSTRIAL APPLICABILITY

The antibodies to intracellular domains of PTPs that are provided by the present invention can bind to an intracellular domain of LAR, or both intracellular domains of CD45 and LAR. These antibodies of the present invention are believed to recognize consensus sequence(s) of phosphatase domains of PTPs, therefore, they are useful for analysis and quantitation of PTPs, for identification and detection of novel PTPs, and for obtaining novel phosphatases by cloning and the like. Furthermore, these antibodies can be extremely useful tools for elucidating signal transduction mechanisms of insulin, and various control mechanisms. Furthermore, the antibodies can be applied for developing useful diagnostic methods of insulin resistance and NIDDM, for prophylaxis and diagnosis of various disease states of syndrome X that is based on insulin resistance, and for prophylaxis and diagnosis of onsets of arteriosclerosis and cardiac diseases.

Add E17